

CRYSTAL STRUCTURES OF bR(D85S) FAVOR A MODEL OF BACTERIORHODOPSIN AS AN HYDROXYL-ION PUMP

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Key words: Bacteriorhodopsin, ion pump, hydroxyl ion

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Abstract

Structural features on the extracellular side of the D85S mutant of bacteriorhodopsin (bR) suggest that wt bR could be an hydroxyl-ion pump. A position between the protonated Schiff base and residue 85 serves as an anion-binding site in the mutant protein, and hydroxyl ions should have access to this site during the O-intermediate of the wt bR photocycle. The guanidinium group of R82 is proposed (1) to serve as a shuttle that eliminates the Born energy penalty for entry of an anion into this binding pocket, and conversely, (2) to block the exit of a proton or a related proton-carrier.

1. Introduction

Bacteriorhodopsin (bR) is a small membrane protein (M_r approximately 27 kD) that is able to use light energy to generate a proton-motive force across the cell membrane [1,2]. While bR is usually described as being an outwardly driven proton pump, it was initially recognized that its biological function could be equally well explained by treating bR as an inwardly driven hydroxyl-ion pump [1]. This ambiguity in mechanism was never resolved due to the lack of an experimental basis for distinguishing between the two alternatives. As the inquiry into the mechanism of ion transport in bR has advanced to atomic resolution, however, the distinction between these two alternative mechanisms has again been raised in the recent literature [3,4].

The transition from the O-intermediate to the bR₅₆₈ resting state, which is the final step in the photocycle, presents one point at which the proton-pumping mechanism and the hydroxyl-ion pumping mechanism differ quite substantially. The key question that can then be posed is whether (1) the proton on D85 is transferred to the “proton release group” on the extracellular side of the protein, as is proposed in the proton-pump model, or (2) the proton on D85 is abstracted by an hydroxyl-ion that enters into an interior anion-binding pocket located between the Schiff base and the side-chain carboxyl group of D85. In other words, the question is, “In which direction does a charged ion move when D85 is deprotonated, and what is the sign of the charge of that ion?”

The unique chemical features that distinguish the O-intermediate from other photointermediates are (1) the retinal chromophore is believed to have returned to the all-trans configuration, (2) the Schiff base is known to be protonated, and (3) residue D85, which first becomes protonated in the L-to-M transition [3], still remains protonated at this stage. Therefore, the primary chemical difference, as opposed to any protein-conformational difference, between the O-intermediate and the bR₅₆₈ resting state is the charge (protonation state) of the side chain of residue 85.

High-resolution structural studies of the D85S mutant of bacteriorhodopsin, bR(D85S), are of special interest because this mutant is likely to adopt a constitutively O-like protein conformation in the anion-free ground-state. The key features that bR(D85S) and the O-intermediate have in common are the protonated state of the Schiff base and the fact that residue 85 is uncharged and yet offers an hydroxyl group that is available for “solvating” a bound anion. The “acid blue” form of bR, in which D85 is protonated due to the low pH [5,6], may also be in a constitutively O-like state, for the same reasons. It is unlikely, however, that well-diffracting crystals of wt bR could be grown at a pH value of 2 or lower, which is needed to populate the protonated, blue state of wt bR at high occupancy.

Both bR(D85S) and the acid-blue wt protein bind chloride ions and pump them across the cell membrane when illuminated [5,6]. These observations, along with the fact that halorhodopsin - a related membrane protein - serves physiologically as a chloride-ion pump, make it natural to consider that wt bR may also be an anion pump, but one that has high specificity for hydroxyl ions.

High-resolution crystal structures of bR(D85S) have been published recently for which the protein is in either the anion-free (blue) state [7,8] or in a purple state with either bromide ion [9] or nitrate ion [8] in the internal binding pocket. The analysis of these structures has so far focused, however, on describing (1) the favorable interactions that this binding pocket offers to either water molecules or to small anions [8], (2) the conformational differences that are observed on the extracellular side of the protein when the O-like, anion-free protein is compared to the resting state, bR₅₆₈ structure [7], (3) the fact that the extracellular side of the protein “returns” to a bR₅₆₈-like conformation when anions are bound adjacent to the protonated Schiff base [9], and (4) the way in which 13-cis photoisomerization is likely to raise the electrochemical potential of a bound anion [9]. In addition to reviewing again the previously described character of the anion-binding pocket in bR(D85S) in comparison to the corresponding site in wt bR, we now consider the role that the side

chain of R82 plays in the structures mentioned above, with special reference to the question of what the sign of the charge and the direction of ion-transport are likely to be in the O-to-bR₅₆₈ transition.

2. Materials and Methods

Each of the bR(D85S) structures discussed in this manuscript have been previously described [7-10] or are currently under review elsewhere. To briefly summarize, halobacterial culture medium (250g/L NaCl, 20g/L MgSO₄•7H₂O, 3.44g/L Sodium Citrate, 2g/L KCl, 0.265g/L CaCl₂•2H₂O, 0.0895g/L FeCl₂•4H₂O, 0.00545g/L MnCl₂•4H₂O, and 10g/L Bacteriological Peptone (Oxoid, New York, NY, USA), adjusted to pH 7.4 with 10N NaOH before autoclaving, was used for growing bR(D85S) mutant-containing strains. Starter cultures were normally grown in the presence of 1µg/ml Novobiocin. Cell membranes were purified by either a step [7,9] or continuous [8] sucrose density gradient centrifugation. Purified membranes were then solubilized by room-temperature incubation in 1.2% octylglucoside dissolved in 0.025 M Na/K phosphate buffer, pH 5.6. The solubilized membrane was then concentrated to 15 mg/ml in 30,000 Da molecular weight cut-off Centricon filters (Millipore, Billerica, MA, USA).

Crystals were grown in 1-monooleoyl-*rac*-glycerol (Sigma, St. Louis, MO, USA) gel by hydrating 10 mg of dry monoolein with ten microliters of protein in a 0.2ml PCR tube. Crystals of bR(D85S) form spontaneously under these conditions while crystals of halide bound bR(D85S) were grown by overlaying the hydrated monoolein with 100µl of the mother liquor consisting of 100mM Sodium Acetate pH 4.6, 200mM KCl, and 10% PEG 4000. Crystals grew to their final size in 3-6 weeks.

In the case of the halide-free structure [7] crystals were harvested from the monoolein gel by physical extraction with a standard nylon cryo-loop (Hampton Research, Aliso Viejo, CA, USA) and plunge frozen directly into liquid nitrogen. Anion-bound crystals were extracted from aliquots of the gel that had been transferred to and partially disrupted by incubating overnight in a lipase

solution consisting of 100mM Sodium Acetate, pH 4.6, 200mM KCl, and 50mg/ml lipase (Type VII, *Candida rugosa*; Sigma, St. Louis, MO, USA). Individual crystals were then transferred to a solution containing no halide, 100mM Sodium Acetate, pH 4.6, 10% PEG 4000 to remove the bound chloride. Soaking in solutions lacking small anions caused the crystals to visibly turn from purple to blue when viewed via a stereo-microscope, indicating that the chloride ion had left the binding site. These blue crystals were then soaked sequentially in cryo-protectant solutions consisting of 100mM Sodium Acetate, pH 4.6, 12%, 16%, 20%, and 25% PEG 4000, respectively and subsequently plunge frozen in liquid nitrogen.

Diffraction data for the halide-free crystals [7] were collected at ALS beamlines 5.0.2 and SSRL beamline 9.1, while data for the anion-soaked crystals was collected at ALS beamline 8.3.1 using a 30 μ m pinhole collimator. Data reduction was performed with the HKL suite of programs [11] and the Elves scripts [12] for the halide-free and anion-bound structures respectively. Data reduction performed by the Elves scripts utilized the programs MOSFLM [13] and SCALA [14]. Molecular replacement using wild-type ground state bacteriorhodopsin as the starting search model, without the retinal, water, and lipid molecules was performed by the program CNS Version 1.1 [15]. Refinement with CNS and model building using the program O [16] together with annealed simulated omit, $|F_o|-|F_c|$, and $2|F_o|-|F_c|$ maps reduced the values of R and R_{free} to their final values.

3. Results and Discussion

The first two questions that we now wish to discuss are (1) whether the anion-binding site in the O-like bR(D85S) mutant is physically adjacent to residue 85, as it is in halorhodopsin [17], and (2) whether the wt bR structure contains a similar site where a hydroxyl ion could hypothetically bind and thus - when bound - abstract a proton from the neutral form of D85. If these conditions were not satisfied, it would be difficult to argue that wt bR might be a hydroxyl-ion pump.

As is shown for bromide ion in Figure 1A and for nitrate ion in Figure 1B, x-ray crystal structures show that small anions do, indeed, bind adjacent to the side chain of residue 85 in bR(D85S). The bound anion also makes a close ion-pair interaction with the protonated Schiff base, which effectively compensates for the energy-cost of burying the anion within a low-dielectric environment [18,19]. The corresponding site in wt bR is occupied by water 402, as is shown in Figure 1C, and a water molecule also occupies this site in bR(D85S) when there is no anion in the binding pocket, as is shown in Figure 1D.

The structural data are therefore compatible with a model in which a hydroxyl ion enters the anion-binding site to produce a transition state between O and bR₅₆₈, for which the anion-bound structures shown in Figures 1A and B might be considered to be stable, ground-state analogs. We emphasize, however, that such a hypothetical structure, were it to occur in the wt O-intermediate, is much more likely to be a transition state than a stable intermediate. Our reasoning is that the transitional ion-configuration $D212^{\cdot-} \dots OH^{\cdot-} \dots D85^0$ would be unstable relative to $D212^{\cdot-} \dots HOH \dots D85^{\cdot-}$, since the former configuration would put two anions into direct, van der Waals contact. The latter configuration, on the other hand, would bridge two anions with a solvating water molecule, as is found to be the case in wt bR₅₆₈. Indeed, formation of the stable analogs of the proposed transition state, shown in Figures 1A and B, requires that D212 be protonated by working at a pH of 5 or lower [8], confirming the intuitive suggestion that the juxtaposition of anions would be a high-energy state even in the presence of the dual, positive counter ions provided by the Schiff base and R82, respectively. We draw attention to the fact that D212 may be in a “protonation equilibrium” with D85 during the O-to-bR transition of wt bR [20] as well as that of E194 and E204 mutants [21], a fact that must ultimately be accounted for by electrostatic calculations for alternative models of the step in which D85 is deprotonated.

The third question that we are able to address with the current high-resolution structural data is whether it is more likely that (1) a proton (or related, positively charged ion) would move from

D85 to the vicinity of E194 and E204, referred to as “the proton release group” [22,23], or (2) a hydroxyl ion would move from this site into the internal, anion-binding site. The fact that the positively charged guanidinium group of R82 blocks the mouth of the only solvent-accessible route between the anion-binding pocket and the extracellular side of the membrane [9] suggests that the latter is mechanistically more likely to be true. The packing of helices on the extracellular side of the membrane appears to be flexible enough to allow multiple, alternative rotamer conformations for this side chain, as is shown in Figure 2. For none of these alternative conformations, however, is there a continuous, solvent-accessible tunnel that goes past the positively charged guanidinium group [9]. As we have pointed out previously [9], the function of this highly flexible, buried guanidinium group might be to “prepay” the Born energy cost when shuttling an anion from the extracellular side of the membrane into the internal anion-binding site. By the same token, however, this same group would impose an additional energy barrier, beyond that of the Born energy itself, for outward cation (i.e. H^+) transport from the site of D85.

If it is proposed, for the sake of argument, that wt bR is an hydroxyl-ion pump, one might well ask why chloride and other anions, which are in excess of one molar concentration in the natural environment, do not competitively inhibit hydroxyl-ion binding and transport. As was implied above, however, binding of a competing anion (e.g. chloride ion) is almost certainly precluded in wt bR at pH values above the pK_a of D212. At pH values low enough to protonate D212, however, small anions such as chloride ion should bind adjacent to the Schiff base in the O-intermediate state of the wt photocycle, just as they do in the resting state when the pH is low enough to constitutively protonate D85. A prediction of this analysis is that the quantum efficiency of “proton” pumping in wt bR should decrease as chloride-ion concentration is increased, but only when the pH is below the pK_a of D212. Conversely, OH^- would not be expected to compete with other anions as a potential charge carrier in bR(D85S) unless the $[OH^-]$ is greater than ~ 10 mM, at

which point other factors resulting from the extremely high pH (e.g. deprotonation of the Schiff base) would interfere with the function of the protein.

An alternative mechanism – how it can work

The following is a step-by-step explanation of how the hydroxyl-ion pump model can account for known biochemical data. The various points emphasized in the following discussion are each represented in figures 3 and 4. Figure 3 gives the reader a structural overview of the location of the key residues that are discussed, while figure 4 shows a schematic diagram of the specific steps discussed below.

Water splitting and hydroxyl-ion binding

This scheme begins by considering the O intermediate to be the initial state in the pumping cycle. In this state, the protein is believed to have an extracellularly open conformation as judged by the x-ray crystal structure of the anion-free form of bR(D85S) [10]. Residues including E194 and E204 comprise, along with water molecules, a complex known as the proton release group (PRG) [24,25], which is likely to be in a deprotonated state at this point. In addition, the positively charged side-chain of R82 adopts an extracellularly facing conformation. At this point, the pump is ready to accept a hydroxyl-ion substrate. However, due to the low dissociation constant of water, a free hydroxyl-ion is not readily available. To overcome this challenge, bR instead binds a neutral water molecule in the region of the PRG where it becomes polarized between the positively charged R82 and the negative charges of E194 and E204. The protein then acts like a water-splitting enzyme, abstracting a proton to protonate the PRG and forming an ion pair between the hydroxyl ion and R82. It is interesting to note that the homologous residue to bacteriorhodopsin's E204 in halorhodospin is a threonine (T230), where the water-splitting function is not needed. In hR it would seem to be advantageous, instead, to reduce the number of negatively charged groups at the entry site in order to increase substrate recruitment. Formation of the ion pair with R82 helps to

lower the Born energy associated with introducing the substrate anion into a region of relatively low dielectric constant [18]. R82, which is suspected to act like a dynamic gate in the halorhodopsin and bR(D85S) anion-pumping schemes, then helps to deliver the hydroxyl-ion into the binding site.

Once delivered to the substrate-binding site, the hydroxyl-ion immediately abstracts a proton from the protonated D85 residue. This reaction produces the neutral water molecule (HOH402) that is seen in x-ray crystal structures between the protonated Schiff base and the negatively charged D85 [26]. Substrate binding then induces a reorganization of hydrogen bonds within the extracellular side of the protein, and the helices close towards the center of the molecule. The resulting state corresponds to the bR resting state in which the hydroxyl-ion substrate has been loaded into the binding site, the extracellular side has closed, and the protein awaits the energy input (a photon absorption event) needed to initiate the transport cycle.

Energy coupling and internal substrate transport

After the absorption of a photon, the retinal isomerizes during the transition from the ground-state to the K-intermediate, taking the chromophore from an initial (resting) *all-trans* configuration to a *13-cis* configuration [27,28]. We speculate, based on evidence from x-ray crystal structures of M-intermediates, that in the transition between the L and the first of the early-M substates, the protonated Schiff base separates from the substrate water molecule (HOH402). The movement of the positively charged Schiff base away from the polarized water molecule changes the local electrostatic environment such that the concerted abstraction of a proton from the water molecule by D85 and abstraction of the Schiff base proton by the resulting hydroxyl-ion becomes possible. Recent NMR data suggests that a species similar to a chloride ion influences the spectrum in the region of the SB at this point [29]. In the hydroxyl-ion pump model, the resulting neutral water molecule, already pulled in the direction of the cytosol by movement of the Schiff base, now would move fully to the cytosolic side of the retinal. Although the earliest known structure of the M

intermediate shows this water molecule moving towards the extracellular side of the protein, relative to its position in the ground-state structure [30], in the structures of several later M intermediates two of the three water molecules — known to initially occupy the binding site — are no longer seen. Rather, two additional water molecules, or new voids sufficiently large to accommodate water molecules, are seen on the cytoplasmic side of the retinal in x-ray crystal structures of early-M intermediates [10,31], suggesting that the two “missing” water molecules may have moved in the direction of the cytoplasm. Furthermore, the neutralization of residue 85 causes R82 to adopt the extracellularly facing conformation which, in the transition between early and late-M states, lowers the pK_a of the PRG. As a result, the proton that was first abstracted from the water molecule during the O-to-bR transition is now released back to the extracellular face of the protein [24,25].

Substrate release

The final step in the transport of the hydroxyl-ion substrate first involves the concerted abstraction of a proton from a water molecule (on the cytoplasmic side) by the then-deprotonated Schiff's base and the concomitant transfer of a proton from the then-protonated D96 onto the hydroxyl-ion, possibly via a network of hydrogen bonded waters. In addition to the increased number of water molecules that are located on the cytoplasmic side of the retinal as a result of the earlier step of substrate transport, the opening of the cytoplasmic face of the protein that occurs between the late-M phase and the N intermediate [24, 25] further increases the level of hydration in the area immediately surrounding residue D96. This increased polarity of the local environment surrounding the protonated D96 residue lowers the pK_a of this side chain sufficiently to allow its deprotonation, with transfer of the proton to the Schiff base [32-35], as described above. As the cytoplasmic channel again closes between the N and O states, the water is squeezed out and the pK_a of residue D96 is raised sufficiently to abstract a proton from one of the departing water molecules, thereby generating a hydroxyl-ion that must exit on the cytoplasmic face. The overall result of this

model of the photocycle is the net transport of one hydroxyl-ion across the plasma membrane and the acidification of the extracellular medium.

4. Conclusions

High-resolution structural data for bR(D85S) in the anion-free and anion-loaded state lend support to the proposal that wt bR is an hydroxyl-ion pump. It is suggested that the mechanistic function of a buried, protonated aspartic acid residue at position 85 is to confer extremely high specificity for binding hydroxyl ions in wt bR, especially at pH values higher than the pK_a for D212. An analysis of the structural data that is currently available thus offers a parsimonious and plausible explanation for why a single point mutation at residue 85 eliminates the ability of bacteriorhodopsin to generate a proton-motive force while at the same time converting the protein into a chloride-ion pumping protein.

The side chain of R82 is structurally well-positioned to assist the entry of anions, including hydroxyl ions, into the binding site that is located between the protonated Schiff base and D85. By the same token, however, the positively charged guanidinium group of R82 is likely to block proton-transfer from D85 to the external side of the membrane. As a result, it would seem that entry of a hydroxyl ion (by the same process that allows entry of small anions in bR(D85S)) represents a simpler model for the mechanism by which D85 is deprotonated in the transition from the O intermediate to the resting state of wt bR. However, distinguishing between any hydroxyl-ion pumping scheme and the proton-pumping model ultimately requires appropriate experimental approaches that are able to exclude one or more of the models. The main obstacle to designing some of the most obvious experiments is the high permeability of neutral water for even the tightest lipid bilayers [36]. This high permeability contributes an overwhelming background flux to measurements of light-induced flow of a labeled “OH” species across the membrane. Therefore, until a convincing experimental or computational approach resolves the

question of whether bR is a proton or hydroxyl-ion pump, we suggest that both hypotheses be considered in the interpretation of new data.

Acknowledgements: This work was supported in part by NIH Grant GM51487.

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Figure Legends:

Figure 1. The polar “ROH” groups of residues S85 and protonated D212, along with the protonated Schiff base of retinal, form an internal binding pocket in bR(D85S) that has affinity for a broad spectrum of small anions [8], as well as water molecules. Anions or water molecules bind in virtually the same locations as follows: **A.** bromide ion, shown as a red sphere, in bR(D85S); **B.** nitrate ion in bR(D85S); **C.** HOH 402 in wt bR, shown as a blue sphere; and **D.** HOH bound adjacent to S85 in bR(D85S) in the absence of small anions. The gold stick and ball residue that fades into the background, at the top of each figure, is the retinal polyene chain, and the ϵ -amino group of K216 forms the protonated Schiff base that interacts from above with each type of ligand. The indole NH group of W86 also points into the binding pocket from behind. The membrane normal is nearly vertical in these figures, with the extracellular side of the membrane toward the bottom.

Figure 2. A representative ensemble of the rotamer conformations of R82 that exist in various high-resolution structures of bR(D85S), wild-type bR, and the E204Q mutant of bR. They are colored as follows: Blue - the halide-free state of bR(D85S); Magenta – the bromide-bound structure of bR(D85S); Orange – a wild-type early-M intermediate; Coral – an early-M intermediate formed by the E204Q mutant; Purple – the ground state of wild-type bR. Note that an upward-facing conformation is favored when, in addition to D212, there is a second anion in the binding site [bromide in bR(D85S) or aspartate anion in wt bR], while a downward-facing conformation is adopted when there is a water molecule in this binding site. These alternative conformations are not always adopted with full occupancy, as is the case when nitrate is in the binding pocket of bR(D85S). Other factors such as an alternative rotamer conformation of Y83 (shown in the figure) or the provision of space for additional water molecules (blue spheres in Figure 1) can influence the preferred conformation of the side chain for R82.

Figure 3. Cartoon representation of proton and hydroxyl-ion movement. Proton movement is shown with red arrows while hydroxyl-ion movement is shown with blue arrows. As a compromise between completeness and clarity, a red arrow from the Schiff base to D85 has been drawn smaller than the others. The difference in size does not mechanistically distinguish this step from any of the others. Key residue side chains are shown overlaid on a faded-ribbon representation of bacteriorhodopsin to orient the reader to the relative positions of the residues within the protein. The actual entry and exit points of either the protons or hydroxyl-ions is not known, and thus the arrows representing these steps should be considered to be merely schematic in nature.

Figure 4. Possible mechanism for hydroxyl ion transport. This figure was adapted from Betancourt and Glaeser [3]. This model demonstrates schematically a sequence of events that would account for the observed biochemical and structural experiments for wild-type bR. Protons and hydroxyl ions are colored to help track the progress of individual atoms through the photocycle. The proton release group is abbreviated by PRG, while numbers and SB indicate individual residues and the Schiff base, respectively. The isomerization state of the retinal is indicated by either cis or trans. Brackets indicate proposed transition states rather than actual intermediates. Subscripts E and L designate early and late substates of the M intermediate, respectively, while M_N designates a protein structure similar to that of the N intermediate, but one in which the Schiff base is still deprotonated, as in the M intermediate.

Figure 1

Figure 2.

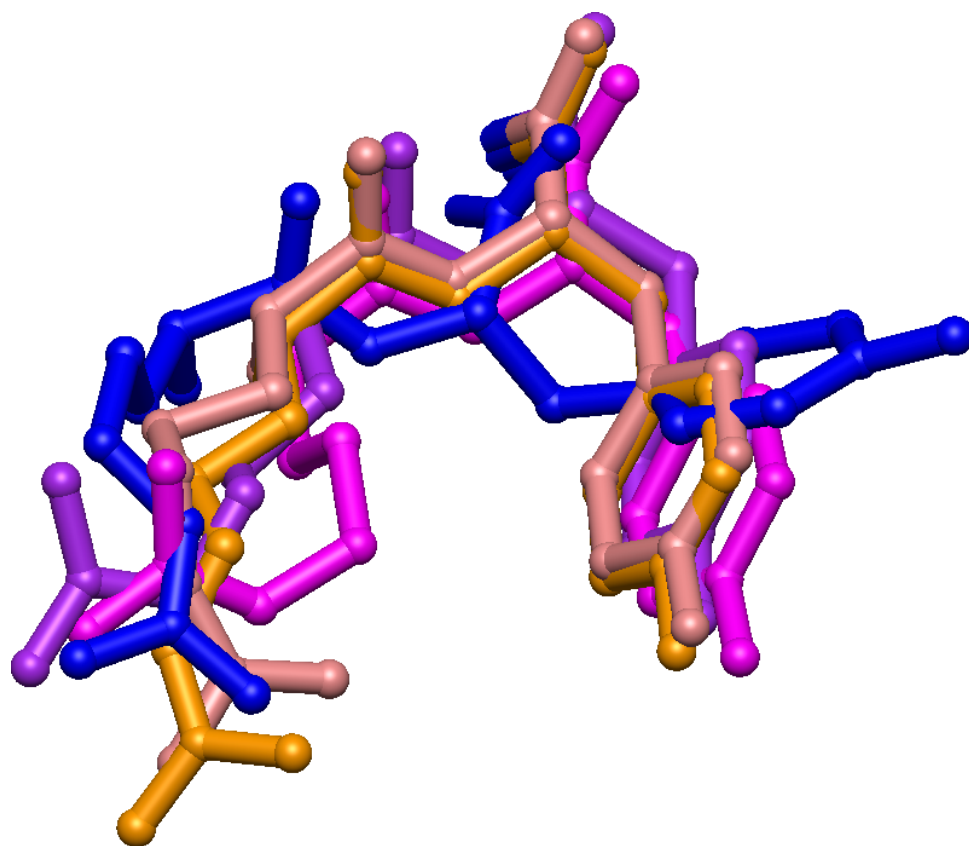


Figure 3.

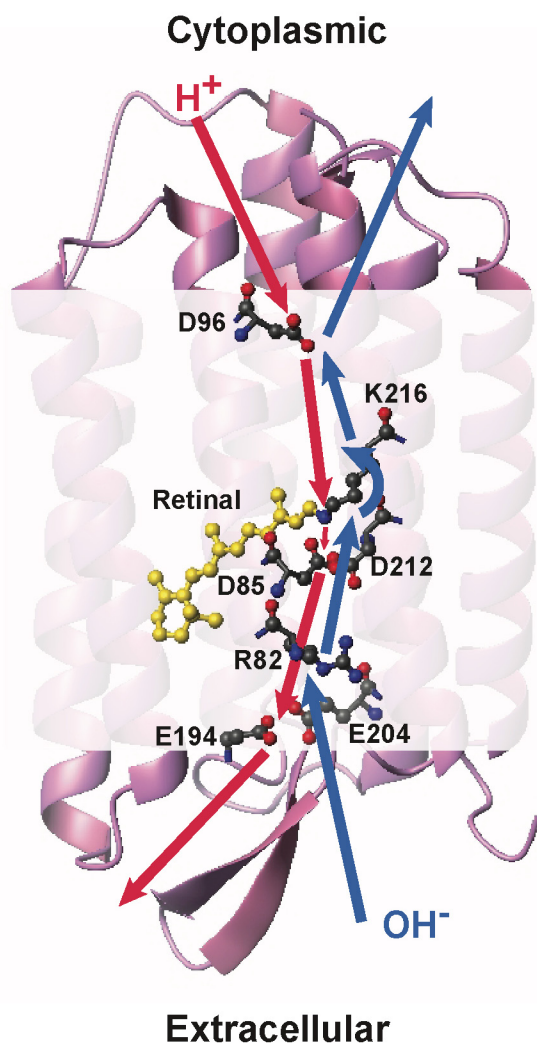


Figure 4.